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Differences in the rDNA-Bearing Chromosome Divide the Asian-Pacific and Atlantic Species of *Crassostrea* (Bivalvia, Mollusca)

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Abstract. Karyotype and chromosomal location of the major ribosomal RNA genes (rDNA) were studied using fluorescence *in situ* hybridization (FISH) in five species of *Crassostrea:* three Asian-Pacific species (*C. gigas, C. plicatula,* and *C. ariakensis*) and two Atlantic species (*C. virginica* and *C. rhizophorae*). FISH probes were made by PCR amplification of the intergenic transcribed spacer between the 18S and 5.8S rRNA genes, and labeled with digoxigenin-11-dUTP. All five species had a haploid number of 10 chromosomes. The Atlantic species had 1–2 submetacentric chromosomes, while the three Pacific species had none. FISH with metaphase chromosomes detected a single telomeric locus for rDNA in all five species without any variation. In all three Pacific species, rDNA was located on the long arm of Chromosome 10 (10q)—the smallest chromosome. In the two Atlantic species, rDNA was located on the short arm of Chromosome 2 (2p)—the second longest chromosome. A review of other studies reveals the same distribution of NOR sites (putative rDNA loci) in three other species: on 10q in *C. sikamea* and *C. angulata* from the Pacific Ocean and on 2p in *C. gasar* from the western Atlantic. All data support the conclusion that differences in size and shape of the rDNA-bearing chromosome represent a major divide between Asian-Pacific and Atlantic species of *Crassostrea.* This finding suggests that chromosomal divergence can occur under seemingly conserved karyotypes and may play a role in reproductive isolation and speciation.

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Abbreviations: CI, centromeric index; FISH, fluorescence *in situ* hybridization; NOR, nucleolar organizer region.

Introduction

Classification and phylogenetic analysis of oysters are problematic because oysters have few informative morphological characteristics. Shell coloration and morphology in oysters are highly variable and sensitive to environmental influence. Anatomy of soft tissue is difficult and provides only limited information. Phylogenetic analyses of oysters may have to rely on a multidiscipline approach using morphological, molecular, and cytogenetic characteristics. Molecular data have been used for phylogenetic analysis of oysters and have shown great promise (Banks *et al.,* 1993; Littlewood, 1994; Ó Foighil *et al.,* 1995, 1998; Ó Foighil and Taylor, 2000; Lapègue *et al.,* 2002). Cytogenetic analysis may provide additional characteristics for phylogenetic comparisons and insight about major genomic changes at chromosome levels. Chromosomal rearrangement and differentiation are important mechanisms for reproductive isolation and speciation in some taxa (White, 1978; King, 1993).

Most of the cytogenetic studies in oysters so far have focused on karyotyping, chromosome banding, and NOR (nucleolar organizer regions) staining in *Ostrea* species (Insua and Thiriot-Quiévreux, 1991; Li and Havenhand, 1997). Studies in *Crassostrea* are scarce and provide little interspecific comparison (Ladrón De Guevara *et al.,* 1996; Leitao *et al.,* 1999a, b). Although oysters have a low haploid number of 10 chromosomes (Nakamura, 1985), oyster chromosomes are small and similar in arm ratios, which makes

karyotypic analysis inherently difficult. Probably due to the small chromosome size and the lack of cell lines required for making elongated chromosomes, chromosome banding in oysters is difficult to obtain and reproduce. C- and G-banding patterns have been produced in three *Crassostrea* species (Rodriguez-Romero *et al.,* 1979; Leitão *et al.,* 1999a), but offered little help in the reliable identification of oyster chromosomes. Ag-NOR staining is also variable and often shows intraspecific variations in number and location, which poses problems for interspecific comparisons (Thiriot-Quiévreux and Insua, 1992; Ladrón De Guevara *et al.,* 1994).

Fluorescence *in situ* hybridization (FISH) is a relatively new technology and now widely used for chromosome identification, gene mapping, localization of gene expression, and studies on chromosome rearrangement in a variety of organisms (Swiger and Tucker, 1996; Nath and Johnson, 1999). By direct DNA base pair hybridization, FISH provides specific and reproducible localization of genes and DNA sequences on chromosomes. Repetitive DNA sequences and genes that are present in high copy numbers and tandem repeats are ideal for use as FISH probes because of their large target size. Unique sequences longer than 80 kb, such as P1 and BAC clones, can be readily mapped to chromosomes by FISH and used as chromosome-specific probes (Jiang *et al.,* 1995). Unique sequences shorter than 1 kb are generally difficult to assign by FISH, although not impossible (Schrimf *et al.,* 1999). Chromosome paint probes have been developed to label specific chromosomes or chromosome regions in some organisms (Rabbitts *et al.,* 1995; Shi *et al.,* 1997). The unambiguous labeling and identification of chromosomes by FISH has made it possible to study chromosome rearrangements in cancer cells and at evolutionary scale.

FISH may provide a solution to the reliable identification of oyster chromosomes, which has not been possible through traditional karyotyping, and permit cross-species comparisons. The technique has recently been used to study oyster chromosomes and shows considerable advantages over traditional methods. Using FISH, a repetitive element has been mapped to centromeric regions of several chromosomes in the Pacific oyster *Crassostrea gigas* (Clabby *et al.,* 1996; Wang *et al.,* 2001), and the vertebrate telomere sequence (TAAGGG)n has been mapped to telomeres of three species of *Crassostrea* (Guo and Allen, 1997; Wang and Guo, 2001). Nine P1 clones have been assigned to specific chromosomes in *C. virginica* (Wang, 2001).

The major ribosomal RNA genes (rDNA), which correspond to NORs, have also been mapped by FISH in three species of *Crassostrea* (Zhang *et al.,* 1999; Xu *et al.,* 2001; Cross *et al.,* 2003). FISH analysis of rDNA provided validation for Ag-NOR staining and eliminated any uncertainty and intraspecific variations. Interestingly, Xu *et al.* (2001) found that the two species of *Crassostrea* studied, one Pacific and one Atlantic species, differ in the size and shape of the rDNA-bearing chromosome. In a species with an Asian-Pacific origin, *C. angulata,* the rDNA-bearing chromosome is the same size and shape as in the Pacific species, but differs from that in the Atlantic species (Cross *et al.,* 2003). To determine if the difference is shared by other species of *Crassostrea,* we used FISH to study the chromosomal location of rDNA in five *Crassostrea* species that are available to us, including the two species studied by Xu *et al.* (2001) but with different populations, two additional Asian-Pacific species (*C. plicatula* and *C. ariakensis*), and one additional Atlantic species (*C. rhizophorae*). Our results plus existing NOR data suggest that differences in the rDNA-bearing chromosome represent a major divide between Asian-Pacific and Atlantic species of *Crassostrea.*

Materials and Methods

Species studied

Five species of *Crassostrea* were included in this study: three Asian-Pacific species (*C. gigas* (Thunberg, 1793), *C. plicatula* (Gmelin, 1791), and *C. ariakensis* (Fujita, 1913)), and two Atlantic species (*C. virginica* (Gmelin, 1791) and *C. rhizophorae* (Guilding, 1828)). *C. gigas* was obtained from a hatchery in Penglai, Shandong, northern China. The *C. gigas* studied by Xu *et al.* (2001) was from a Rutgers stock originated from Washington State. The use of different stocks was intended to detect possible variation among populations. *C. plicatula* was collected from Qingdao, Shandong, northern China. *C. ariakensis* was collected from Yangjiang, Guangdong, southern China. *C. virginica* was from two sources: wild oysters from Delaware Bay and hybrids between Delaware Bay wild and a hatchery stock (NEH, the same stock used by Xu *et al.,* 2001) maintained at the Haskin Shellfish Research Laboratory (HSRL), Rutgers University, New Jersey. The hatchery stock, which originated from Long Island Sound, has been maintained at HSRL for over 10 generations (selected for disease-resistance). *C. rhizophorae* was the first-generation progeny of a Caribbean population produced and maintained at the Harbor Branch Oceanographic Institute, Ft. Pierce, Florida.

Chromosome preparation

For *C. plicatula* and *C. ariakensis,* chromosome metaphases were prepared from gill tissue. Oysters were incubated in 0.005% colchicine in seawater for 8–10 h. Gill tissues from five oysters of each species were dissected and treated with the hypotonic solution 0.075 *M* KCl for 30 min before being fixed in freshly prepared Carnoy's fixative (3:1 methanol/acetic acid, v:v). The fixative was changed twice, and fixed samples were stored at 4 °C. Metaphases of *C. gigas, C. virginica,* and *C. rhizophorae* were made from early embryos according to the protocol described by Xu *et*

al. (2001). For embryo production, 3 females and 2 males of *C. gigas*, 4 females and 4 males of *C. virginica*, and 6 females and 3 males of *C. rhizophorae* were used.

Slides were prepared using an air-drying technique. Gill tissues were chopped into fine pieces and resuspended in freshly made fixative. Two or three drops of cell (from gills) or embryo suspension were loaded onto a clean slide and flooded with two drops of 1:1 methanol/acetic acid. Slides were air-dried and stored at −20 °C until FISH analysis.

Probe construction

Oyster genomic DNA was prepared from adductor muscle of *C. gigas* and *C. virginica* according to Doyle and Doyle (1987). Intergenic transcribed spacers between the 18S and 5.8S RNA genes (ITS1) were amplified, labeled by PCR incorporation of digoxigenin-11-dUTP, and used as FISH probes. Primers, 5′-GGTTTCTGTAGGTGAACCTGC and 5′-CTGCGTTCTTCATCGACCC, were designed using conserved sequences flanking the ITS1. ITS1 was used as the FISH probe so that the primer sequences were conserved and could allow universal amplification, while the internal sequences were variable and might permit species-specific detection. PCR amplification was conducted in 25 μl of a PCR mixture containing PCR buffer with 1.5 m*M* of $MgCl_2$, 0.4 mg/ml of BSA, 0.2 m*M* each of dATP, dCTP, and dGTP, 0.13 m*M* of dTTP, 0.07 m*M* of digoxigenin-11-dUTP, 0.5 U of Taq DNA polymerase, 1 μ*M* of each primer, and 1 μg of oyster genomic DNA. Digoxigenin-11-dUTP and other PCR reagents were purchased from Roche (Indianapolis, IN). PCR was performed in a DeltaCycler II system thermal cycler (ERICOMP Inc., San Diego, CA) with 30 cycles of 1 min of denaturing at 95 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C, and final extension at 72 °C for 5 min. PCR products were verified on 2% agarose gels. DIG-labeled PCR products were purified using G-50 columns (Roche) before being used as FISH probes.

Fluorescence in situ *hybridization*

Separate ITS1 probes were made for *C. gigas* and *C. virginica*, using their respective genomic DNA as templates. Both probes were tested for FISH in all five species. FISH was conducted according to protocols described by Xu *et al.* (2001). Before FISH, slides were stained with Leishman's stain for 3–5 min and screened for metaphases. Negative controls in which the FISH probe was replaced with distilled water were included to detect possible nonspecific hybridization. FISH signals were observed under a Nikon epi-fluorescence microscope equipped with a CCD camera and imaging system.

Chromosomes were measured for the calculation of relative length (RL) and centromeric index (CI), and classified according to criteria defined by Levan (1964). Ten metaphases were measured for each species. The CI of each chromosome represents the mean and standard deviation of the 10 metaphases. When the CI of a chromosome plus and minus the standard deviation overlapped two chromosome categories, the chromosome was designated with labels for two categories. Chromosomes were paired by length and arm ratio, and named 1 to 10 from the longest to the shortest.

Results

A diploid number, $2n = 20$, was found in all five oysters studied. Each karyotype consisted of 10 pairs of metacentric and sometimes submetacentric chromosomes. Karyotype analysis showed that all five species shared a similar karyotype (Table 1). The only noticeable difference between the three Pacific and two Atlantic species was that *C. virginica* and *C. rhizophorae* had 1–2 chromosomes that were clearly submetacentric, while the three Pacific species had no chromosomes that could be unquestionably defined as submetacentric. There were chromosomes in both Pacific and Atlantic species whose centromeric indexes overlapped ranges for metacentric (0.500–0.375) and submetacentric (0.374–0.250) chromosomes. These were classified as metacentric/submetacentric (m/sm in Table 1) chromosomes and not treated as submetacentric chromosomes. One chromosome in *C. virginica* and two chromosomes in *C. rhizophorae* were clearly submetacentric.

PCR amplification of ITS1 in *C. gigas* generated a single fragment of approximately 520 bp in length. Incorporation of digoxigenin-11-dUTP shifted the size of the PCR product to about 670 bp. The PCR product of the same primer pair in *C. virginica* was about 500 bp, slightly shorter than that from *C. gigas*. Both *C. gigas* (*Cg*) and *C. virginica* (*Cv*) probes were used for FISH analysis.

FISH with both *Cg* and *Cv* probes produced positive signals in all five species (Fig. 1). Two bright signals were detected in all metaphases analyzed in all five species without any variation (Table 2). The FISH signals were restricted to one locus (one pair of chromosomes) with no or little background signal elsewhere on the chromosomes. For interphase nuclei, the number of FISH signals varied between one and two. About 50%–68% of nuclei clearly had two FISH signals, while others had one or overlapping signals. In all five species studied, *Cg* and *Cv* probes produced identical results in the number and location of signals, but signal strength differed. In Pacific species, the signals produced by *Cg* probes were generally stronger than those produced by *Cv* probes; conversely, in Atlantic species, *Cv* probes usually produced stronger signals. Only FISH results with the *Cg* probe for Pacific species and the *Cv* probe for Atlantic species are presented in Figure 1. No FISH signal was observed in the negative controls.

Karyotype analysis of FISH signals showed that, in all

Table 1

Karyotype analysis of 10 metaphases in five Crassostrea *species*

Species chromosome	Relative length (mean ± SD)	Centromeric index (mean ± SD)	Classification[1]
C. gigas			
1	12.43 ± 0.25	0.41 ± 0.01	m
2	11.86 ± 0.55	0.46 ± 0.02	m
3	10.97 ± 0.40	0.45 ± 0.02	m
4	10.50 ± 0.16	0.43 ± 0.01	m
5	10.28 ± 0.42	0.38 ± 0.03	m/sm
6	9.88 ± 0.14	0.41 ± 0.01	m
7	9.43 ± 0.22	0.45 ± 0.02	m
8	9.29 ± 0.25	0.40 ± 0.03	m/sm
9	8.66 ± 0.22	0.41 ± 0.03	m
10	7.61 ± 0.53	0.42 ± 0.02	m
C. plicatula			
1	12.87 ± 0.37	0.46 ± 0.01	m
2	11.10 ± 0.27	0.41 ± 0.02	m
3	10.83 ± 0.31	0.46 ± 0.02	m
4	10.35 ± 0.29	0.41 ± 0.01	m
5	10.14 ± 0.49	0.45 ± 0.02	m
6	9.62 ± 0.05	0.45 ± 0.02	m
7	9.59 ± 0.24	0.39 ± 0.02	m/sm
8	9.09 ± 0.36	0.39 ± 0.02	m/sm
9	8.45 ± 0.38	0.39 ± 0.01	m
10	7.79 ± 0.28	0.40 ± 0.02	m
C. ariakensis			
1	12.03 ± 0.47	0.38 ± 0.02	m/sm
2	11.53 ± 0.39	0.46 ± 0.01	m
3	10.95 ± 0.59	0.40 ± 0.01	m
4	10.51 ± 0.37	0.41 ± 0.02	m
5	9.90 ± 0.81	0.45 ± 0.03	m
6	9.77 ± 0.35	0.46 ± 0.01	m
7	9.31 ± 0.44	0.42 ± 0.01	m
8	9.05 ± 0.22	0.40 ± 0.02	m
9	8.89 ± 0.32	0.44 ± 0.02	m
10	8.13 ± 0.56	0.39 ± 0.01	m
C. virginica			
1	12.51 ± 0.37	0.47 ± 0.01	m
2	11.64 ± 0.26	0.38 ± 0.01	m/sm
3	10.96 ± 0.29	0.41 ± 0.01	m
4	10.81 ± 0.23	0.47 ± 0.01	m
5	10.20 ± 0.31	0.40 ± 0.02	m
6	9.68 ± 0.30	0.47 ± 0.1	m
7	9.41 ± 0.25	0.40 ± 0.02	m
8	8.91 ± 0.22	0.41 ± 0.01	m
9	8.28 ± 0.21	0.35 ± 0.02	sm
10	7.61 ± 0.38	0.47 ± 0.01	m
C. rhizophorae			
1	12.23 ± 0.59	0.46 ± 0.02	m
2	12.22 ± 0.42	0.38 ± 0.01	m/sm
3	11.22 ± 0.39	0.40 ± 0.02	m
4	10.90 ± 0.16	0.46 ± 0.02	m
5	10.09 ± 0.49	0.32 ± 0.04	sm
6	9.96 ± 0.53	0.40 ± 0.01	m
7	9.45 ± 0.57	0.46 ± 0.01	m
8	8.70 ± 0.28	0.47 ± 0.02	m
9	7.83 ± 0.40	0.30 ± 0.03	sm
10	7.38 ± 0.65	0.46 ± 0.01	m

[1] m = metacentric chromosomes; sm = submetacentric chromosomes; m/sm = metacentric or submetacentric, centromeric indexes overlapping two categories.

three Pacific species (*C. gigas, C. plicatula,* and *C. ariakensis*), the FISH signals occurred on the long arms of Chromosome 10 (10q, Fig. 1, Table 2). The assignment was unambiguous because Chromosome 10 was clearly the smallest chromosome in all three species. In all three Pacific species, the signals were found at telomeric regions of the long arm. In the two Atlantic species (*C. virginica* and *C. rhizophorae*), however, FISH signals were found on the short arms of Chromosome 2 (2p, Fig. 1, Table 2). Chromosome 2 was the second longest chromosome in both species and was clearly distinguishable from Chromosome 1 and 3 by its centromeric index. Chromosome 2 in both Atlantic species had a centromeric index of 0.38 (m/sm), compared with 0.46–0.47 for Chromosome 1 and 0.40–0.41 for Chromosome 3 (Table 1). As in the Pacific species, FISH signals were restricted to telomere regions. Karyotypic alignments of the five *Crassostrea* species are presented in Figure 1F.

For both *C. gigas* and *C. virginica,* there was no intraspecific variation in the number and chromosomal location of the rDNA locus among oysters collected from different populations, as demonstrated by results from this study and Xu *et al.* (2001).

Discussion

Fluorescence in situ *hybridization validates nucleolar organizer regions, but not always*

This study provides unambiguous chromosomal assignment of rDNA in five species of *Crassostrea,* including different populations of two previously studied species, *C. gigas* and *C. virginica.* The number and location of the rDNA locus revealed by FISH are clear and without any variation. Despite the use of oysters from different populations, FISH results from this study agree with previous FISH analyses in both *C. gigas* and *C. virginica* (Zhang *et al.,* 1999; Xu *et al.,* 2001). FISH results presented here confirm that rDNA or the major rRNA genes are located at the NOR sites previously reported for *C. gigas* (Thiriot-Quiévreux and Insua, 1992). For *C. rhizophorae,* Lapègue *et al.* (2002) observed one NOR on the short arms of Chromosome 3, which corresponds to the rDNA loci we found on Chromosome 2. Chromosome 3 in Lapègue *et al.* (2002) appeared to be the second longest in the karyotype and had a centromeric index close to that of Chromosome 2 in our study.

For *C. virginica* and *C. ariakensis,* however, FISH results are in conflict with results from Ag-NOR staining. Leitão *et al.* (1999b) reported two NOR sites for *C. virginica* (Chromosomes 1 and 5) and *C. ariakensis* (Chromosomes 9 and 10) with considerable variation, while FISH in this study detected only one rDNA locus in the two species. Our FISH results are clear, consistent, and supported by early studies in *C. virginica* (Zhang *et al.,* 1999; Xu *et al.,* 2001). The

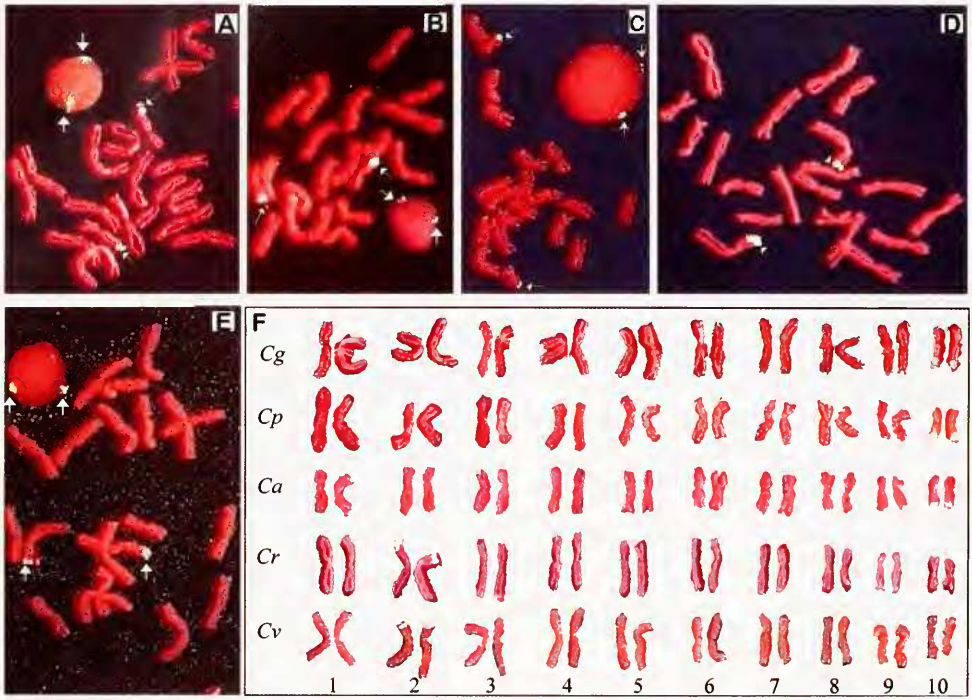

Figure 1. Fluorescence *in situ* hybridization (FISH) signals and chromosomal location of the major rRNA genes in five species of *Crassostrea*. (A) *C. gigas* rDNA probe on *C. gigas* chromosomes; (B) *C. gigas* probe on *C. plicatula*; (C) *C. gigas* probe on *C. ariakensis*; (D) *C. virginica* probe on *C. rhizophorae*; (E) *C. virginica* probe on *C. virginica*; (F) chromosome alignment of five species. Arrows show FISH signals.

discrepancy between the FISH and NOR data may be caused by either false-positive Ag-NOR staining or by a lack of correspondence between NORs and the major rRNA genes. Ag-NOR staining targets transcriptionally active NOR sites and is known to produce variable and inconsistent results in the number and sometimes the location of NORs within the same species (Insua and Thiriot-Quiévreux, 1993; Li and Havenhand, 1997). Also, Ag-NOR staining may not be able to separate major (18S-5.8S-28S) from minor (5S) rDNA and other actively transcribed genes. One of the two signals described by Leitão *et al.* (1999b) seems to correspond to the major rDNA locus in our study, but the nature of the other site is unknown. Based on preliminary FISH data (Wang and Guo, unpubl.), the extra NOR site in *C. virginica* is not the site of 5S rDNA. Clearly, Ag-NOR staining can accurately detect major rRNA genes sometimes—as shown by results from *C. gigas*, *C. rhizophorae*, and *C. angulata* (Thiriot-Quiévreux and Insua, 1992; Lapègue *et al.*, 2002; Cross *et al.*, 2003), but not always—as exemplified in *C. virginica* and *C. ariakensis*.

Table 2

Fluorescence in situ *hybridization with* C. gigas *and* C. virginica *rDNA probes on interphase nuclei and metaphase chromosomes in five species of* Crassostrea

Probe/species	% Nuclei with 2 signals (*n*)	% Metaphases with 2 signals (*n*)	Chromosomal location[1]
C. gigas probe			
C. gigas	60 (25)	100 (28)	10q
C. plicatula	60 (25)	100 (10)	10q
C. ariakensis	64 (14)	100 (10)	10q
C. virginica	66 (15)	100 (10)	2p
C. rhizophorae	67 (24)	100 (10)	2p
C. virginica probe			
C. gigas	50 (14)	100 (14)	10q
C. plicatula	55 (18)	100 (10)	10q
C. ariakensis	52 (15)	100 (13)	10q
C. virginica	65 (27)	100 (16)	2p
C. rhizophorae	68 (40)	100 (19)	2p

[1] All locations are telomeric.

Confirmation of NOR results by FISH is necessary. NOR sites, often two per genome, have been reported in several species of *Ostrea* (Insua and Thiriot-Quiévreux, 1991, 1993; Thiriot-Quiévreux and Insua, 1992; Li and Havenhand, 1997). It would be interesting to know if the NOR sites correspond to the major rRNA gene locus in the *Ostrea* species.

This study provides the first report on the karyotype of *C. plicatula*, whose taxonomic status is uncertain at this time. *C. plicatula* (formerly *Ostrea plicatula*) is commonly used to refer to a type of small oyster found in intertidal areas along most of China's coast (Wang *et al.*, 1993). Some believe that *C. plicatula* is the same species as *C. gigas* (Li and Qi, 1994; Yang *et al.*, 2000); others suggest that it is closely related to *C. ariakensis* (Yu *et al.*, 2003); and still others consider it an unresolved *Crassostrea* species and use the name of *Crassostrea* sp. instead (Xu, 1997). *C. plicatula* seems to be a different species from *Alectryonella plicatula* found in southern China (Li and Qi, 1994).

Differences between Pacific and Atlantic species

Results of this study clearly demonstrate that the major rDNA is located on the long arms of Chromosome 10 (the smallest) in all three Asian-Pacific species and on the short arms of Chromosome 2 (the second longest) in the two Atlantic species. A critical examination of NOR data indicates that the same pattern holds true in two other species: NORs (or the major rRNA genes, pending verification by FISH) are located on the long arms of Chromosome 10 in the Asian-Pacific species *C. sikamea* and on the short arms of Chromosome 2 in the eastern Atlantic (African) species *C. gasar* (Leitão *et al.*, 1999b; Lapègue *et al.*, 2002; Table 3). The Portuguese oyster *C. angulata* provides a unique and interesting case, adding support to the observed pattern. Both Ag-NOR staining and FISH have indicated that rDNA is located on the long arm of Chromosome 10, the same as in Pacific species (Leitão *et al.*, 1999b; Cross *et al.*, 2003). *C. angulata* is found along the northeastern coasts of the Atlantic and has been assumed to be native there. However, there are conflicting views on the taxonomic status and origin of this species. Some, on the basis of morphological and allozyme data, have suggested that *C. angulata* is the same species as the Pacific oyster *C. gigas* (Menzel, 1974; Buroker *et al.*, 1979). The prevailing view is that *C. angulata* is an Asian species that was introduced to Europe from Japan, although it is possible that *C. gigas* is an Atlantic species that was introduced to Japan from Portugal (Menzel, 1974). More recent analyses using mtDNA sequence data suggest that *C. angulata* is of Asian origin (likely from Taiwan), and that it is closely related, but not identical, to *C. gigas* from Japan (Boudry *et al.*, 1998; Ó Foighil *et al.* 1998). Whether *C. angulata* is the same species as *C. gigas* requires further study, but it is clear from molecular data that *C. angulata* is an Asian-Pacific species.

Another karyotypic difference between Asian-Pacific and Atlantic members of *Crassostrea* is the number of SM chromosomes. In this study, we observed one in *C. virginica*, two in *C. rhizophorae*, and none in the three Pacific species. Despite some variation, other studies also found more SM chromosomes in Atlantic than in Pacific species (Leitão *et al.*, 1999b; Lapègue *et al.*, 2002; Cross *et al.*, 2003). The karyotypic differences between all three Atlantic and five Pacific *Crassostrea* species studied so far are

Table 3

Summary of karyotypic differences in the rDNA-bearing chromosome and the number of submetacentric (SM) chromosomes between Asian-Pacific and Atlantic species of Crassostrea[1]

Species	rDNA location	Number of SM chromosomes: This study	Leitão *et al.* (1999b)	Lapègue *et al.* (2002)	Cross *et al.* (2003)	Average
Pacific species						
C. gigas	10q	0	0			0
C. ariakensis	10q	0	1			0.5
C. plicatula	10q	0				0
C. sikamea	10q[2]		0			0
C. angulata[3]	10q		1		0	0.5
Atlantic species						
C. virginica	2p	1	2			1.5
C. rhizophorae	2p	2		4		3.0
C. gasar	2p[2]		4	4		4.0

[1] Only chromosomes with a centromeric index + SD less than 0.375 were considered to be submetacentric for standardized comparisons.
[2] Based on Ag-NOR staining (Leitão *et al.*, 1999b; Lapègue *et al.*, 2002), pending verification by FISH.
[3] *C. angulata* is considered to be an Asian-Pacific species (Ó Foighil *et al.*, 1998; Boudry *et al.*, 1998).

summarized in Table 3. Differences in the rDNA-bearing chromosome and the number of SM chromosomes represent a major divide between Pacific and Atlantic members of the genus. Oysters are thought to have highly conserved karyotypes. All *Crassostrea* studied so far (about 13 species) have a haploid number of 10 chromosomes and similar karyotypes (Nakamura, 1985). The size and shape of the rDNA-bearing chromosome and the number of SM chromosomes are the first two clearly recognizable chromosomal divergences among species of *Crassostrea.*

There are likely other karyotypic differences between the Pacific and Atlantic species, and between this and other studies for the same species. We did not attempt to match individual chromosomes across species and studies. Chromosome identification in oysters is difficult because oyster chromosomes are similar in size and arm ratio. The accuracy of chromosome measurements varies considerably depending on the degree of chromosomal condensation, the quality of metaphases, and the staining methods used. Chromosome pairing, classification, and naming are prone to errors. In the absence of chromosome-specific FISH probes, chromosome alignment across species is not reliable in oysters and must be viewed with caution. However, difficulties in chromosome identification do not affect the conclusions of this study. The rDNA-bearing chromosomes in the Pacific and Atlantic species are strikingly and consistently different, and the difference in the number of SM chromosomes is independent of chromosome identity and supported by other studies.

The findings of this study suggest that chromosomal divergence among *Crassostrea* species is possible under a seemingly conserved karyotype. The divergence in karyotype is not surprising, and phylogenetic analysis using molecular data has shown that Pacific and Atlantic species of *Crassostrea* form two clades on phylogenetic trees (Littlewood, 1994; Ó Foighil *et al.,* 1998; Ó Foighil and Taylor, 2000; Lapègue *et al.,* 2002). Compared with molecular data, the rDNA-bearing chromosome provides a clear and simple divide between the two species groups, which may represent a single event of macroevolution at the chromosome level or accumulation of chromosome changes over time.

Divergence in karyotype can arise from chromosomal deletion, duplication, translocation, inversion, fission, fusion, and aneuploidy (White, 1978; King, 1993). In our case, the only recognizable difference so far is the size and shape of the rDNA-bearing chromosomes. It is not clear whether Chromosome 10 of the Pacific species is homologous to Chromosome 2 of the Atlantic species. If it is, the divergence may be caused by chromosomal duplications or deletions. If the two chromosomes have little homology other than in the rDNA regions, translocation would likely be responsible.

Chromosomal divergence and hybridization barrier

Major chromosomal divergence can cause reproductive isolation and speciation, by altering normal gene expression and regulation or causing problems for meiosis and fertility in hybrids (White, 1978; King, 1993; Noor *et al.,* 2001; Rieseberg, 2001). Biologically, there is a well-documented postzygotic hybridization barrier between Asian-Pacific and Atlantic species of *Crassostrea* (see review in Gaffney and Allen, 1993). Hybridization within the two geographic species groups produces viable offspring (Wang and Liu, 1959; Zhou *et al.,* 1982; Menzel, 1987; Allen and Gaffney, 1993). Hybridization between *C. virginica* and *C. gigas* or *C. ariakensis* results in high levels of fertilization and apparently normal larval development, but all hybrid larvae die within 2 weeks and before metamorphosis (Allen *et al.,* 1993). By demonstrating significant chromosomal divergence across the hybridization barrier, we raise the possibility that the chromosomal divergence may contribute to the formation of the barrier. Geographic isolation and genic mutations may have played important roles in the speciation of oysters. We present the chromosomal divergence hypothesis as a possible explanation for the postzygotic hybridization barrier between Atlantic and Asian-Pacific species of *Crassostrea,* while recognizing that the barrier may as well be genic. Additional data are needed to discriminate between the two hypotheses. There are about 15 extant members of *Crassostrea,* and most of them live in the Asian-Pacific region (Carriker and Gaffney, 1996). A survey of all species in the genus may reveal karyotypic variation within the geographic ranges. Species from the eastern Pacific and Indian Oceans would be most interesting. Similar studies in the oyster genera *Ostrea* and *Saccostrea* may provide insight into the phylogenetic relationships among the three major groups of Ostreidae.

Finally, this study provides the first chromosomal assignment by FISH of the major rRNA genes in *C. plicatula, C. ariakensis,* and *C. rhizophorae.* The unambiguous mapping of rDNA by FISH made it possible to identify major karyotypic differences between Asian-Pacific and Atlantic species of *Crassostrea.* Results of this study show that FISH is a powerful tool for cytogenetic analysis, especially in species where chromosome identification by traditional methods is challenging. Cytogenetic analysis in most marine invertebrates has been limited primarily due to difficulties of chromosome identification. The application of FISH techniques and development of chromosome-specific probes may enable chromosome identification and phylogenetic comparisons of molluscs and other marine invertebrates.

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